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# Locus NMB0035 codes for a 47-kDa surface-accessible conserved antigen in *Neisseria*

**Summary**. A 47 kDa neisserial outer-membrane antigenic protein (P47) was purified to homogeneity and used to prepare polyclonal anti-P47 antisera. Protein P47 was identified by MALDI-TOF fingerprinting analysis as the hypothetical lipoprotein NMB0035. Two-dimensional diagonal SDS-PAGE results suggested that, contrary to previous findings, P47 is not strongly associated with other proteins in membrane complexes. Western blotting with the polyclonal monospecific serum showed that linear P47 epitopes were expressed in similar amounts in the 27 *Neisseria meningitidis* strains tested and, to a lesser extent, in commensal *Neisseria*, particularly *N. lactamica*. However, dot-blotting assays with the same serum demonstrated binding variability between meningococcal strains, indicating differences in surface accessibility or steric hindrance by other surface structures. Specific anti-P47 antibodies were bactericidal against the homologous strain but had variable activity against heterologous strains, consistent with the results from dot-blotting experiments. An in-depth study of P47 is necessary to evaluate its potential as a candidate for new vaccine designs. [**Int Microbiol** 2006; 9(4):273-280]

**Key words:** *Neisseria meningitidis* · *Neisseria lactamica* · surface antigens · conserved antigens · outer-membrane proteins

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## Introduction

*Neisseria meningitidis* is a gram-negative human parasite whose outer membrane is surrounded by a polysaccharidic capsule that is essential for its pathogenicity and which protects meningococcal cells. Capsular polysaccharide and proteins located in the outer membrane are the main surface antigens of this microorganism. However, environmental and host factors cause a high variability in the antigenicity of these structures, producing a great number of antigenic variants [14,27,28]. In addition to their variable capsular types, meningococci carry out capsular switching, which represents an important mechanism for immune evasion and limits the effectiveness of existing vaccines [33,34].

Meningococcal meningitis and sepsis are severe processes, with mortality rates of about 10% but as high as 50% in the absence of appropriate diagnoses and treatment. Polysaccharidic vaccines developed in the 1960s confer immunity against serogroups A, C, Y, and W135 in adults. In infants and children, good control over disease produced by serogroup C was recently obtained with vaccines based on capsular polysaccharide chemically conjugated to carrier proteins [2]. Serogroup A is the main cause of epidemics in underdeveloped countries, and conjugated vaccines (similar to that against serogroup C) are expected to effectively pre-

vent disease caused by this serogroup. Nevertheless, this conjugation approach is not feasible for infection with serogroup B meningococci, which has very high mortality rates in developed countries [13]. Thus, research carried out over the last few years has focused on the development of vaccines based on outer-membrane proteins (OMPs), with the goal of obtaining effective protection against disease produced by serogroup B infection. While some attempts have involved multiple antigenic variants [17], clinical assays have shown that vaccines based on outer-membrane vesicles (OMVs) elicit bactericidal antibodies and protection. However, they are only effective against homologous strains because the response is mainly directed against PorB and PorA (the serotype and serosubtype antigens), which present high antigenic variability. Although these vaccines confer low immunogenicity in children and generate only short-term responses, they may nonetheless be useful for the control of specific outbreaks of serogroup B and in hyperendemic areas where the number of antigenic variants is limited [21,26]. While several other OMPs produce more effective vaccines [6,7,23], a good candidate has not been found to date. The OMP NadA induces bactericidal activity, but the gene coding for this protein is present in only 50% of the strains [6]. Other OMPs, such as PorB3 or NspA, are present in all strains but their expression or surface accessibility differs between strains [19,20].

**Table 1**. Characteristics of the *Neisseria* strains used in this study

| Strain[1] | Serogroup and serotype[2] | Origin | Clonal complex |
|---|---|---|---|
| *N. meningitidis* | | | |
| B16B6[r] | B:2a:P1.5,2 | Cerebrospinal fluid | ST8 complex, cluster A4 |
| GLD[i] | C:2a:P1.5 | Cerebrospinal fluid | ST11complex, ET37 |
| NMB[i] | B:2b:P1.2,5 | Cerebrospinal fluid | ST11 complex, ET37 |
| M7[nc] | B:2b:P1.2,5 | Cerebrospinal fluid | ST8 complex, cluster A4 |
| Nm9[i] | C:2b:P1.2,5 | Cerebrospinal fluid | ST8 complex, cluster A4 |
| Nm16[i] | B:4:P1.15 | Cerebrospinal fluid | ST8 complex, cluster A4 |
| Nm17[i] | C:2b:P1.2,5 | Blood | ST32 complex, ET5 |
| Nm20[i] | C:2b:P1.2,5 | Cerebrospinal fluid | ST8 complex, cluster A4 |
| Nm21[i] | C:2b:P1.2,5 | Blood | ST8 complex, cluster A4 |
| Nm22[i] | B:15:P1.16 | Cerebrospinal fluid | ST8 complex, cluster A4 |
| Nm30[i] | C:2b:P1.2,5 | Cerebrospinal fluid | ST32 complex, ET5 |
| Nm39[i] | B:2b:P1.2,5 | Blood | ST8 complex, cluster A4 |
| Nm40[i] | B:2b:P1.2,5 | Blood | ST8 complex, cluster A4 |
| Nm59[i] | C:NT:P1.3 | Cerebrospinal fluid | ST22 complex |
| Nm60[i] | B:4:P1.15 | Blood | ST32 complex, ET5 |
| Nm62[i] | HA:2a:P1.5 | Cerebrospinal fluid | ST11 complex, ET37 |
| Nm63[i] | B:12:P1.19,15 | Cerebrospinal fluid | ST32 complex, ET5 |
| Nm68[i] | B:15:P1.19,15 | Blood | ST32 complex, ET5 |
| Nm70[i] | B:4:P1.1 | Cerebrospinal fluid | ST32 complex, ET5 |
| NmP0[c] | W135:NT:P1.3,6 | Oropharynx | ST22 complex |
| NmP2[c] | B:4:P1.3 | Oropharynx | ST22 complex |
| NmP3[c] | B:2a:P2,5 | Oropharynx | ST22 complex |
| NmP5[c] | B:NT:P1.3,6 | Oropharynx | ST198 complex |
| NmP7[c] | B:4:P1.3,6 | Oropharynx | ST254 complex |
| NmP17[c] | W135:NT:P1.3 | Oropharynx | ST11complex, ET37 |
| NmP18[c] | W135:NT:P1.3 | Oropharynx | ST174 ** |
| NmP19[c] | C:15:NT | Oropharynx | ST32 complex, ET5 |
| *N. lactamica* | | | |
| NlP3[c] | NA | Oropharynx | |
| NlP5[c] | NA | Oropharynx | |
| NlP6[c] | NA | Oropharynx | |
| NlP9[c] | NA | Oropharynx | |
| *N. sicca* | | | |
| NsP4[c] | NA | Oropharynx | |
| *N. perflava* | | | |
| NpfP0[c] | NA | Oropharynx | |

[1]NT, nontypeable; HA, highly agglutinable; NA, not applicable.

[2]Based on six genes matched. The only difference is a change in the *adk* gene from allele 5 (ST174 complex) to allele 16. ST-1251 is recorded in the MLST database with no clonal assignment.

[r]Reference strain; [i]invasive strain, [nc]non-capsulated mutant of NMB, [c]carrier strain

The most important challenge in producing an effective meningococcal vaccine based on OMPs is to find an antigen or a group of antigens common to all strains and able to induce long-term protective immune responses both in adults and children. Previous studies in our laboratory demonstrated an immunological response in convalescent patients against an antigenic neisserial OMP (P47) inducible by iron restriction, and its antigenic cross-reactivity in a limited number of neisserial strains [35]. The aim of the present work was to purify and characterize P47 as well as to evaluate its antigenic conservation and ability to elicit bactericidal responses.

## Materials and methods

**Bacterial strains and culture conditions.** The characteristics of all strains used are listed in Table 1. *N. meningitidis* strains GLD and B16B6 were kindly provided by P. Borriello (Queen's Medical Center, University Hospital, Nottingham, UK); strain NMB, by D. Granoff (Children's Hospital, Oakland Research Institute, Oakland, CA, USA); and strain M7 (a capsule-deficient mutant of NMB), by M. Pizza (Istituto di Ricerca Immunobiologica di Siena, Siena, Italy). All other strains were from our laboratory. Strains were maintained and cultured under iron-restricted conditions as described previously [22].

**Multilocus sequence typing**. Multilocus sequence typing (MLST) of each strain was done at the Instituto Carlos III (Madrid, Spain) by determination of the allelic profiles obtained from a 450-bp internal region in the *abcZ*, *adk*, *aroE*, *fum*, *gdh*, *pdhC*, and *pgm* genes (Table 1). Assignation to clonal complexes was done by comparing the profiles with those in the meningococcal MLST database [http://pubmlst.org/neisseria/].

**Extraction and purification of the P47 protein**. OMPs were extracted from strain *N. meningitidis* Nm30 using Triton X-114. Cells from iron-restricted cultures were recovered, washed with 20 mM Tris-HCl, 500 mM NaCl, pH 7.5 (TSB), and suspended (0.1 g/ml) in chilled (4ºC) TSB containing 0.02% (w/v) sodium azide and 4% (v/v) Triton X-114. The suspension was incubated overnight at this temperature in a water bath with 200 rpm constant shaking and then centrifuged at 10,000 ´*g* at 4ºC for 30 min to remove cellular debris. The supernatant was incubated at 37ºC for 3 h to provoke phase separation, and the detergent and aqueous phases were separately recovered (discarding the interface). Proteins present in both phases were identified by SDS-PAGE and Western blotting using an antiserum from mice immunized with homologous OMVs. P47 was purified by elution after separation on 4–15% gradient SDS-polyacrylamide gels, essentially as described by Hager et al. [11]. Gel debris was discarded and the proteins were precipitated with trichloroacetic acid [3]. Protein purity was checked by SDS-PAGE, Western blotting with anti-OMV mouse sera, and MALDI-TOF peptide-map fingerprinting analysis of bands cut from silver-stained gels.

**SDS-PAGE and Western blotting**. SDS-PAGE was carried out according to standard protocols, using 12% polyacrylamide gels and the discontinuous buffer system described by Laemli [15]. For the analysis, bacterial cells were fixed by adding 1% (v/v) formaldehyde to the culture just after growth, followed by incubation at room temperature for 30 min with 100 rpm constant shaking. The cells were then washed twice with phosphate-buffered saline (PBS; 0.1 M sodium phosphate buffer, pH 7.2, containing 0.1 M NaCl), resuspended at $10^9$ cells per ml in PBS containing 0.05 mM phenylmethylsulfonyl fluoride (PMSF), and maintained at 4ºC until needed. For some experiments, the cells were permeabilized with 70% ethanol [19] and/or treated with chloroform vapor [24] prior to fixation. Samples containing 15 µl ($1.5 \times 10^7$) of formalin-fixed bacteria were mixed with double-strength sample buffer and heated to 95°C for 30 min before loading. After separation of the bands, the gels were stained with copper [16] for visualization of the proteins, destained, and transferred to a polyvinylidine difluoride (PDVF) membranes for Western blotting. Membranes were blocked, incubated overnight at 37ºC with antisera (1:1000 working dilution) and then with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulins or HRP-conjugated goat anti-mouse immunoglobulins. The immune complexes were visualized with 4-chloro-1-naphthol. Analysis of the antigens and calculation of the molecular weight of the protein of interest were done using Quantity One sofware (Bio-Rad Laboratories, Spain).

**Diagonal electrophoresis.** SDS-PAGE consisting of 7.5–15% gradient gels and standard electrophoretic settings was used for both the first and second dimension in diagonal electrophoresis as described previously [30].

**Antisera**. Mouse sera against OMVs were obtained as described before [31]. Rabbit sera against P47 and OMVs from strain Nm30 were obtained by modification of a protocol described by Vaitukaitis [36]. Briefly, 2-kg New-Zealand rabbits were immunized by either multiple intradermal injection with 500 µg of OMVs or 13 mg of purified P47, in 500 µl of Freund's complete adjuvant. On day 54, the rabbits were injected intramuscularly with the same amounts of immunogen in 100 µl of PBS, and two more doses were administered at 8-day intervals. Rabbits were bled by cardiac puncture 12 days after the last injection and the sera obtained were heat-inactivated and stored at –20ºC in 250-µl aliquots. Non-immune sera were obtained from animals injected with Freund's adjuvant (without any antigen) following the same immunization schedule.

**Dot-blotting assays**. For dot-blot enzyme assays [8], $1.5 \times 10^6$ formalin-fiexed cells (1.5 µl bacterial suspension) were spotted onto nitrocellulose membranes (0.45 µm , Bio-Rad, Richmond, CA, USA) and allowed to dry at room temperature. The membranes were then incubated with blocking solution (TBS with 5% Blotto, Bio-Rad, USA) at room temperature, washed three times with TBS-Tween (0.05% Tween-20 in TBS), and then probed with the same sera used for Western blotting. Accessibility was tested in some of the membranes by treating them with chloroform vapors for 20 min and then probing them with antisera. Negative controls consisted of 1.5 µl of a solution containing 2 mg bovine serum albumin (BSA)/ml in TBS-Tween.

**Bactericidal activity**. Bactericidal assays were carried out as previously described, using *N. meningitidis* strains Nm30 and B16B6 for the tests, and human serum as source of complement (adsorbed with each target strain and tested with non-immune mouse serum to confirm the absence of non-specific killing) [29]. Negative controls without immune serum, complement, or both were included, and at least two assays, with three replicates each, were carried out on different days. Bactericidal activity is expressed as the mean percentage reduction in the number of colony-forming units (CFUs) with respect to those obtained in experiments with non-immune mouse serum. CFU reductions of more than 50% were considered indicative of significant bactericidal activity [12].

## Results

Cultivation of *N. meningitidis* strain Nm30 under conditions of iron restriction strongly induced the production of P47 protein. After the OMPs were solubilized with Triton X–114, the protein partitioned into the detergent phase (Fig. 1). When solubilized in electrophoresis sample buffer, the band-

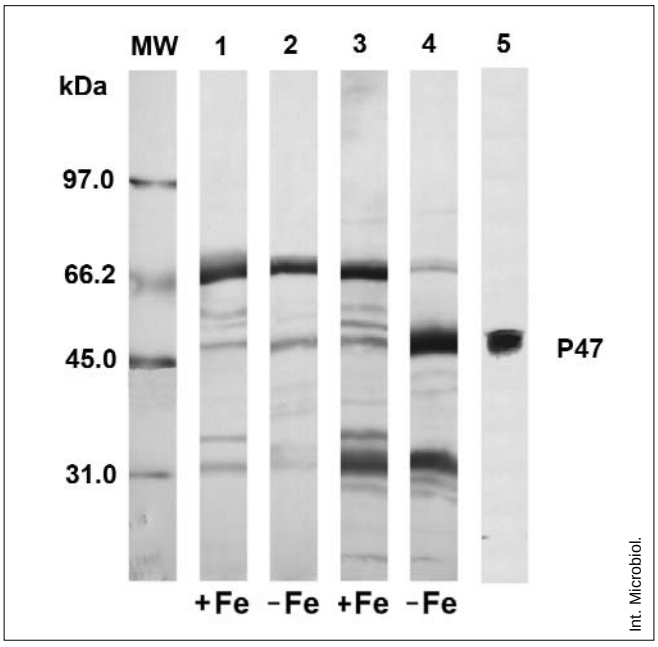


**Fig. 1.** Western blots of outer-membrane proteins (OMPs) from strain *Neisseria meningitidis* Nm30 cultured in normal (+Fe) or iron-restricted (–Fe) media and solubilized with Triton X-114. The proteins were separated by SDS-PAGE and probed with homologous anti-OMV mouse serum. Lanes: A and B, aqueous phases; C and D, detergent phases; E, purified P47 protein. MW, molecular mass markers (kDa).

ing pattern of P47 overlapped with that of a minor antigenic protein of similar molecular mass that was not inducible by iron restriction (not shown). Purification by elution from SDS-polyacrylamide gels of the detergent-phase extracts yielded highly purified, homogeneous P47, as shown by SDS-PAGE, Western blotting with homologous anti-OMV mouse sera, and MALDI-TOF peptide-map fingerprinting analysis (Table 2). Accordingly, this protein was identified with the products of genes NMA0281 (*N. meningitidis* strain Z2491, serogroup A), NMB0035 (*N. meningitidis* strain MC58, serogroup B), NMC65c08 (*N. meningitidis* strain FAM18, serogroup C), and NG02050 (*N. gonorrhoeae* strain FA1090).

To determine whether P47 formed complexes with other OMPs, diagonal electrophoresis of these proteins from strain Nm30 was carried out under non-denaturing conditions in the first-dimension run, and either non-denaturing (Fig. 2A) or denaturing (Fig. 2B) conditions in the second-dimension run. Some proteins formed complexes that could be resolved by heat treatment (spots under the diagonal) or whose mobility was heat-dependent (spots over the diagonal). The corresponding Western blots, probed using anti-P47 serum (Figs. 2C,D), showed that the 47-kDa protein did not form complexes with other OMPs, since it appeared only in the diagonal regardless of the analytical condition.

The expression of P47 in *N. meningitidis* and the commensal neisserial strains tested as well as the cross-reactivity of the anti-P47 antibodies were analyzed in Western blots using a rabbit serum specific for the protein (Fig. 3). P47 was detected in all 33 strains analyzed, although reactivity with anti-P47 rabbit serum was slighty lower in *N. lactamica* strains and much lower in the other commensal strains of *Neisseria* (Fig. 3A). The antiserum did not cross-react with any other protein in the Western blots. Moreover, the reactivity of the serum with formalin-fixed whole cells in dot-blots was highly variable and did not correlate with in the results

**Table 2.** MASCOT search results obtained by MALDI-TOF analysis of purified P47 of *Neisseria meningitidis**

| Observed | Mr (expected) | Mr (calculated) | Delta | Start–end | Miss | Peptide |
|---|---|---|---|---|---|---|
| 1034.60 | 1033.59 | 1033.54 | 0.05 | 145–153 | 0 | LSQPLADYK |
| 1058.68 | 1057.67 | 1057.62 | 0.05 | 77–84 | 1 | KLEWEILK |
| 1150.64 | 1149.63 | 1149.64 | –0.00 | 124–134 | 1 | GKLVVTDSGFK |
| 1157.65 | 1156.64 | 1156.61 | 0.03 | 262–272 | 0 | EIDALAFPPGK |
| 1203.59 | 1202.59 | 1202.55 | 0.04 | 349–358 | 1 | TKDGFETYDK |
| 1413.87 | 1412.87 | 1412.84 | 0.03 | 314–325 | 0 | IVDLFRPLIEAK |
| 1541.96 | 1540.95 | 1540.93 | 0.02 | 313–325 | 1 | IVDLFRPLIEAK |
| 1544.84 | 1543.84 | 1543.81 | 0.03 | 273–288 | 0 | VVGGASELIEEVAGSK |
| 1735.93 | 1734.92 | 1734.92 | 0.00 | 232–246 | 1 | IEYALWVEKDVSGVK |
| 1797.00 | 1795.99 | 1795.98 | 0.01 | 366–382 | 0 | ALQASINALAEDLAQLR |
| 1883.88 | 1882.87 | 1882.83 | 0.04 | 296–312 | 0 | YSHTDLSDFQANVDGSK |
| 1928.98 | 1927.97 | 1927.97 | 0.01 | 85–102 | 1 | GVMVVDERENIAPGLSDKYSHTDLSDFQANVDGSK |
| 1944.99 | 1943.98 | 1943.96 | 0.02 | 85–102 | 1 | GVMVVDERENIAPGLSDKM |
| 2027.05 | 2026.04 | 2026.06 | –0.02 | 196–213 | 0 | IEPIAELFSELDPVIDAR |
| 2052.01 | 2051.01 | 2051.01 | 0.00 | 126–144 | 1 | LVVTDSGFKDTANEADLEK |
| 2670.25 | 2669.24 | 2669.18 | 0.06 | 289–312 | 1 | ISGEEDRYSHTDLSDFQANVDGSK |

*Mass: 42396; score: 203; expect: 9.6e-015; peptides matched: 16
Identification: conserved hypothetical protein NMB0035 (*Neisseria meningitidis* MC58)

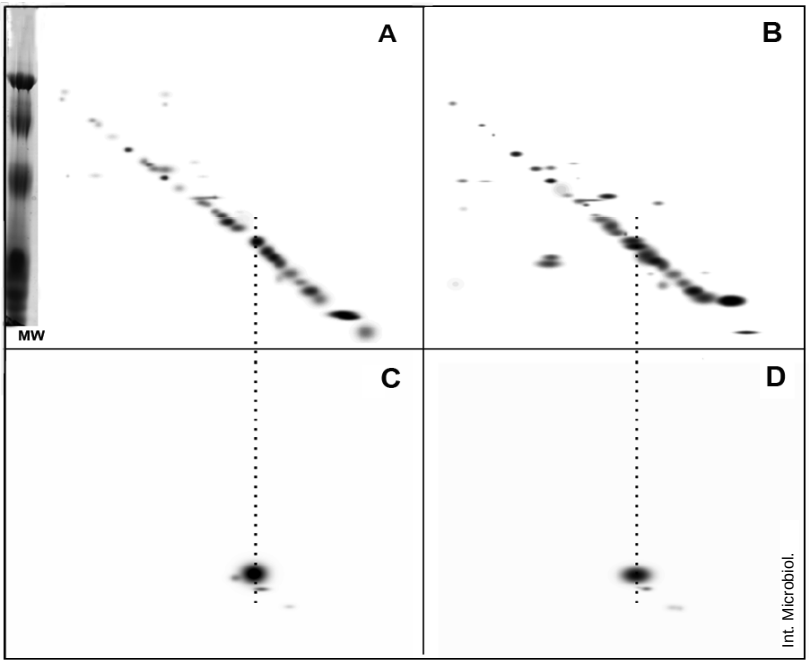


**Fig. 2.** Two-dimensional diagonal electrophoresis of OMPs from *Neisseria meningitidis* Nm30. The first dimension was run with samples treated at ambient temperature in all cases, and the second dimension after treatment at ambient temperature (A, C) or 95°C (B, D). Gels were either stained with Coomassie blue (A, B) or the proteins were transferred to PVDF membranes and further analyzed by Western blotting with anti-P47 sera (C, D).

of the Western blots (Fig. 3B). This suggested that P47 is masked in whole cells by capsular material. To test this possibility, cells were permeabilized with ethanol prior to fixation with formaldehyde and then analyzed by dot-blotting. The permeabilized cells did not show an increase in the accessibility of P47 to antibodies, and, for some strains, reactivity was even lower in the ethanol-treated cells. Delipidation with chloroform also failed to increase the surface exposure of P47 (not shown).

Bactericidal activity assays showed that anti-P47 serum had significant bactericidal activity against the homologous bacterial strain (Nm30), with 77% killing compared to the control. However, the efficacy of the antiserum against the four heterologous strains tested was variable, ranging from a

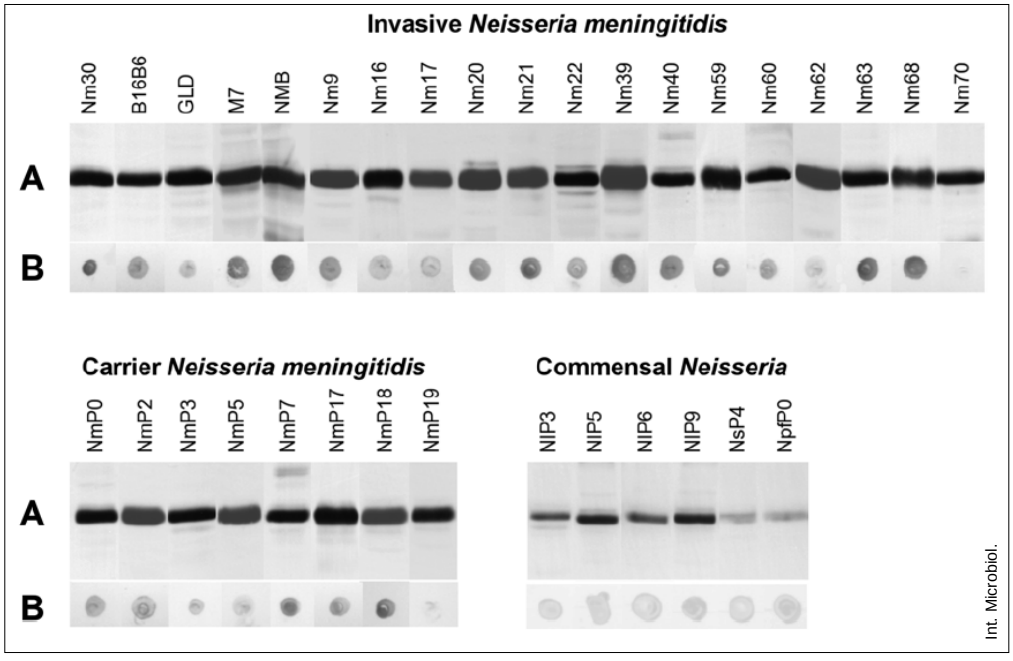


**Fig. 3.** (**A**) Western blots of OMPs from *Neisseria meningitidis* and commensal neisserial strains separated by SDS-PAGE and probed with a rabbit antiserum raised against purified P47. (**B**) Reactivity of the antiserum with formalin-fixed cells in dot-blotting assays.

minimum of 2% to a maximum of 42%. Bactericidal activity against other strains was not measured as it was not possible to remove the natural killing activity from the human serum used as the complement source.

## Discussion

´The development of vaccines effective against serogroup B meningococci is a major challenge for researchers in this field. Antigens suitable for effective vaccines should be highly conserved, easily accessible at the bacterial surface, and able to elicit long-lasting bactericidal responses. Currently, the most promising approaches are based on bacterial OMPs, which are being investigated by several strategies, including reverse vaccinology [1], heterologous commensal strains [10], and multiple PorA expression mutants [18]. In previous studies, we identified a meningococcal, outer-surface iron-repressible protein that showed high antigenic conservation among meningococcal strains [35].

Attempts to purify P47 by excision from SDS-PAGE gels, after separation of OMPs isolated from iron-restricted meningococcal cultures and solubilized with SDS (electrophoresis sample buffer), resulted in the co-purification of P47 with another protein of similar molecular mass. The latter protein was detected by MALDI-TOF analysis and identified as the translation elongation factor Tu (*N. meningitidis* MC58; GenBank accession number NP_273197). The use of Triton X-114 for the solubilization of OMPs resulted in the partition of P47 into the detergent phase and allowed its purification to homogeneity, as verified by MALDI-TOF analysis. P47 homologues in annotated meningococcal genome databases [http://www.tigr.org] are described as a "predicted periplasmic lipoprotein involved in iron transport" (NMB0035), or as a "putative lipoprotein containing N-terminal signal sequence and appropriately positioned PS00013 prokaryotic membrane lipoprotein lipid attachment site" (NMA0281). The partition of P47 largely in the detergent phase strongly suggests its membrane localization [5].

Strain *N. meningitidis* Nm30 was chosen for purification of P47 due to its high expression of the protein after cultivation under iron-restricted conditions. The purification procedure was also tested in other strains, and a highly purified product was obtained from each of them. The rabbit serum resulting from immunization with purified P47 was highly specific in Western blotting assays, did not react with any other OMP, and cross-reacted completely with all *Neisseria* strains tested. The faint bands visible in some strains were probably due to non-specific reactions because they did not appear in the overstained control of the homogenous product obtained during purification. The reactivity of the antiserum was slightly lower with *N. lactamica* strains and much lower with the other commensal species, which could have been due to lower P47 expression levels or to differences in the accessibility of P47 at the surfaces of different bacterial species.

Formalin-fixed bacterial cells were used to avoid possible alterations in the bacterial cell surfaces (and in the accessibility of surface components) arising from the drying step in the dot-blotting procedure. Results obtained with anti-P47 sera and formalin-fixed cells were identical to those using live intact cells (data not shown) and demonstrated the accessibility of P47 at the bacterial surface, although it revealed differences both among *N. meningitidis* strains and between *N. meningitidis* and the commensal strains of *Neisseria*. These differences suggested substantial variability in the expression, surface accessibility, or epitope arrangement of P47 in the different strains. Western blotting showed that reactivity with the anti-P47 serum was through linear epitopes, and differences between strains were not very apparent (except for the commensal *Neisseria*). These results suggested that the reason for the inter-strain variability observed in the dot-blotting experiments was the accessibility and/or epitope arrangement of P47.

Antigen masking in *N. meningitidis* by surface structures, such as the capsule and lipopolysaccharides (LPSs), has been reported [24,25]. Ethanol treatment removes removes neisserial capsular polysaccharide and LPS, exposes surface antigens, and thus facilitates their accessibility to antibodies [19]. In *N. meningitidis*, the capsular structure and/or the amount and length of lipooligosaccharides (LOSs) can significantly vary between strains. Most commensal *Neisseria* lack a capsule and have specific LOSs due to the absence of *lgt* genes (LOS glycosyltransferase) in most species [14,28–32].

The possibility that binding of anti-P47 antibodies to ethanol-treated and untreated cells was sterically hindered was investigates in a capsulated strain (NMB) and its non-capsulated mutant (M7). Ethanol has been reported to damage the outer structures of bacteria, partially eliminating the capsule and LOS outer residues, thereby exposing surface proteins to antibodies [14]. Our results showed no significant effects of ethanol or chloroform on P47 exposure to antibodies, whereas treatment with both reagents abolished reactivity in the two commensal *Neisseria* strains tested. Such effects could have been due to the effective removal of reactive antigenic residues, which, in turn, was facilitated by the lower expression of P47 and/or by differences in the surface composition (lack of capsule and different LOS) of the commensals. Other authors have noted no effect of the capsular material on surface antigen accessibility [4], which, together with

our results, suggests that LOS are responsible for the differences in the antibody accessibility of P47.

Bactericidal activity was measured in only a limited number of strains due to the impossibility of removing the natural bactericidal activity of human serum (used as the complement source) against other strains. Sera from agammaglobulinemic individuals, which would have been the best choice for these kinds of experiments, were not available. Nonetheless, a certain degree of correlation between bactericidal activity and the reaction intensity in dot-blot experiments was observed. An exception was strain Nm9, which reacted with the antiserum to an extent similar to the reaction of B16B6, even though it showed a much lower bactericidal activity. It has to be taken into account that the intensity in dot-blotting experiments is due to the binding of any antibody subclass, whereas bactericidal activity is related only to the binding of bactericidal subclasses; thus, the two measurements do not necessarily correlate.

A recent study by Giuliani et al. [9] demonstrated a highly efficient (>78%) vaccine formulation containing five genome-derived antigens, which confirms that protein-based vaccines are good alternatives for serogroup-independent immunization against *N. meningitidis*. The present work showed the antigenic cross-reactivity of P47 in a great number of *N. meningitidis* strains and in some commensal *Neisseria*, particularly *N. lactamica*, as well as its surface exposition and the bactericidal activity of anti-P47 antibodies. Work in progress is focused on the analysis of sequence variability in the *p47* gene in all strains, determination of surface exposure of the protein by flow cytometry, and analysis of the bactericidal activity of anti-P47 sera in a larger number of strains. The results should help to determine the antigenic variability of P47 and the applicability of this protein in future vaccine designs.

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## El *locus* NMB0035 codifica un antígeno de superficie conservado de 47 kDa en *Neisseria*

**Resumen**. El tratamiento con Triton X-114, seguido de la separación por SDS-PAGE permitió la purificación de una proteína antigénica de 47 kDa (P47) de la membrana externa de *Neisseria* de forma homogénea, lo que permitió la preparación de sueros policlonales anti-P47. La P47 se identificó como la lipoproteína (hipotética) NMB0035 mediante el análisis de la huella molecular realizado por MALDI-TOF. Los resultados obtenidos por SDS-PAGE diagonal bidimensional sugieren que, a diferencia de otros resultados previos, la P47 no parece estar fuertemente asociada a otras proteínas en complejos de membrana. Análisis de inmunoelectrotransferencia (*Western blot*) empleando el suero policlonal monoespecífico demostró que los epítopos lineales de la P47 se expresaron de forma similar en las 27 cepas de *Neisseria meningitidis* ensayadas y, aunque con menor intensidad, también lo hicieron en algunas *Neisseria* comensales, especialmente en *N. lactamica*. Sin embargo, resultados obtenidos por transferencia de mancha con el mismo suero demostraron que entre las cepas meningocócicas existe variabilidad en la capacidad de unión, lo que sugiere que o bien hay diferencias en la accesibilidad en superficie o bien una ocultación estérica producida por otras estructuras de superficie. Los anticuerpos específicos anti-P47 tenían efecto bactericida contra la cepa homóloga pero mostraron una actividad variable contra cepas heterólogas, lo cual concuerda con los resultados de la transferencia de mancha, y hace pensar que es necesario estudiar esta proteína más profundamente para evaluar su potencial como candidata para nuevos diseños de vacunas. [**Int Microbiol** 2006; 9(4):273-280]

**Palabras clave**: *Neisseria meningitidis* · *Neisseria lactamica* · antígenos de superficie · antígenos conservados · proteínas de la membrana externa

## O *locus* NMB0035 codifica um antígeno de superfície conservado de 47 kDa em *Neisseria*

**Resumo**. O tratamento com Triton X-114, seguido da separação por SDS-PAGE permitiu a purificação de uma proteína antigênica de 47 kDa (P47) de *Neisseria* de forma homogênea, o que possibilitou a obtenção de soros policlonais anti-P47. A P47 foi identificada por MALDI-TOF como a lipoproteína (hipotética) NMB0035. Os resultados obtidos por SDS-PAGE diagonal em duas dimensões sugerem que, ao contrario de resultados prévios, a P47 não parece estar estreitamente associada a outras proteínas em complexos de membrana. Análise de transferência de Western empregando o soro policlonal monoespecífico mostram que os epitopos lineares da P47 se expressaram de forma similar nas 27 cepas de *Neisseria meningitidis* ensaiadas e, mesmo que com menor intensidade, também o fizeram em algumas *Neisseria* comensais, especialmente em *N. lactamica*. No entanto, resultados obtidos por dot blotting com o mesmo soro demonstraram que entre as cepas meningocócicas existe variabilidade na capacidade de união, o que sugere que há diferenças na acessibilidade em superfície ou uma ocultação estérica produzida por outras estruturas de superfície. Os anticorpos específicos anti-P47 foram bactericidas contra a cepa homóloga mas mostraram uma atividade variável contra cepas heterólogas, o que coincide com os resultados de dot blotting, indicando que é necessário realizar estudos desta proteína em maior profundidade para avaliar seu potencial como candidato a novos desenhos de vacinas. [**Int Microbiol** 2006; 9(4):273-280]

**Palavras chave**: *Neisseria meningitidis* · *Neisseria lactamica* · antigenos de superficie · antigenos conservados · proteínas da membrana externa